Effects of Estrogen on Atherosclerosis Formation and Serum Nitrite/Nitrate Concentrations in Cholesterol-fed Ovariectomized Rabbits

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This study was to examine the effects of estrogen replacement on atherosclerosis formation in ovariectomized cholesterol-fed rabbits. We also examined serum levels of nitrite/nitrate, stable metabolites of nitric oxide, to investigate the involvement of nitric oxide. Female New Zealand White rabbits were ovariectomized and divided into 3 groups; 1) fed a normal diet (ND group, n = 5), 2) fed a 1% cholesterol diet (CD group, n = 6), or 3) fed a 1% cholesterol diet and received estrogen replacement (CD + E group, n = 7). After 3 months, the rabbits were sacrificed to examine atherosclerosis formation. Atherosclerosis was not observed in ND. The oil red O-positive area in the aorta was significantly greater in CD than in CD + E (CD, 17.3 ± 2.2; CD + E, 9.3 ± 0.8%, p < 0.05). Stenosis of the coronary artery was also significantly greater in CD than in CD + E (CD, 30.6 ± 9.7; CD + E, 6.7 ± 2.9%, p < 0.05). There was no significant difference in serum lipids between CD and CD + E. Serum nitrite/nitrate levels were significantly lower in CD than in ND (ND, 37.6 ± 3.6; CD, 25.3 ± 3.1 μM, p < 0.05). There was a non-significant trend towards higher nitrite/nitrate levels after estrogen replacement (CD + E, 34.4 ± 3.8 μM, p = 0.08 vs. CD). These results suggest that direct actions on vascular wall including nitric oxide production contribute to the antiatherogenic effects of estrogen.

Key words: Hormone replacement, Nitric oxide, Ovariectomy

The incidence of cardiovascular diseases is lower in premenopausal women than in men of a similar age, and increases markedly after the menopause (1, 2). Estrogen replacement therapy is effective in protecting postmenopausal women from coronary heart disease (2-4). These epidemiological findings suggest that estrogen has an anti-atherogenic action. The mechanism, however, is not fully understood.

A number of investigators have reported that lipid metabolism contributes to the effects of estrogen (5, 6). Estrogen increases serum high-density lipoprotein (HDL) cholesterol level and decreases serum low-density lipoprotein (LDL) cholesterol level. Also, direct actions of estrogen on the vascular wall have been reported. Estrogen has both endothelium-independent (7, 8) and endothelium-dependent (9-11) vasodilator actions, which may contribute to the inhibition of atherosclerosis. Estrogen-induced production of nitric oxide (NO) (12, 13) is considered to be a possible explanation for endothelium-dependent vasodilation and cardioprotection. However, we can find no report investigating the anti-atherogenic effects of estrogen and estrogen-induced NO production in the same study.

The aim of this study was to examine the effects of estrogen replacement on atherosclerosis formation in ovariectomized cholesterol-fed rabbits, and to further
examine serum levels of nitrite/nitrate (NOx), stable metabolites of NO, in order to investigate the involvement of NO in the effects of estrogen.

Materials and Methods

Animal experiments

Eighteen female New Zealand White rabbits weighing about 3.0 kg each were purchased from the Saitama Animal Laboratory (Saitama). They were kept individually in stainless steel cages in a room maintained at 23°C. All surgical and experimental procedures were approved by the Animal Research Committee of the University of Tokyo.

The rabbits were bilaterally ovariectomized under pentobarbital anesthesia and 10,000 unit/kg penicillin G was administered intramuscularly. After a one-week recovery period, they were randomly divided into three groups: 1) normal diet (ND, n = 5), 2) 1% cholesterol diet (CD, n = 6), and 3) 1% cholesterol diet plus estrogen replacement (CD+E, n = 7). Estrogen was replaced by subcutaneous injection of 20 μg/kg estradiol dipropionate (Teikoku Hormone Co., Tokyo) once a week. Cholesterol was added to the normal diet (RC4, Oriental Yeast, Tokyo), which contained 3% fat. The calorie count was 294 kcal per 100 g. The rabbits were allowed free access to water. Each rabbit received 120 g daily of the assigned diet and was fed the whole diet every day of the experiment.

Body weight and systolic blood pressure were measured at 0.1 and 3 months of the study. Systolic blood pressure was measured noninvasively using a device (Oiso Ikakikai, Tokyo) previously described (14). We also reported that blood pressure measured by this method well correlated with that measured by direct cannulation (14). At 3 months into the study, blood (10 ml) was collected from the ear vein of each animal after 16-hour fasting. Blood samples were centrifuged at 1,500 g for 10 min at 4°C. The serum was stored at −20°C for chemical analysis and NOx measurement. After blood sampling, rabbits were exsanguinated under pentobarbital anesthesia.

Serum total cholesterol and triglyceride were measured enzymatically, and HDL cholesterol was measured by heparin-Ca²⁺ Ni²⁺ precipitation method (15). Serum levels of thiobarbituric acid-reactive substances (TBARS) were measured as the indices of lipoprotein oxidation (16). Serum estradiol concentration was measured by sensitive radioimmunoassay.

Quantification of atherosclerosis in the aorta and coronary artery

Aortas were excised from the aortic root to the bifurcation. Surrounding adventitial tissues were removed and the aortas were longitudinally divided into anterior and posterior halves of approximately the same size. The

posterior half of each aorta was used for the quantification of atherosclerosis as described previously (14, 17). The aortas were attached to a plastic board and were fixed with 10% formalin buffer for 24 h. The aortas were then washed with distilled water and stained by incubation in Oil red O solution for 20 min as described by Willis et al. (18). After staining, the intimal surface was photographed. The area of the stained surface was measured with a digitizer connected to a Macintosh computer. The ratio of the stained area to the whole surface of the posterior half of the aorta (% Oil red O-positive area) was considered to be the magnitude of atherosclerosis.

Hearts were also excised and fixed with 10% formalin buffer for 24 h. The left main coronary artery approximately 2 mm distal from the ostium was used for the evaluation of coronary atherosclerosis. Three serial cross-sections at 0.5-mm intervals were prepared and stained by Elastica van Gieson staining. The area inside the internal elastic lamina (hypothetical luminal area) and the area of the intima were measured with a digitizer connected to a Macintosh computer. The ratio of the intimal area to the hypothetical luminal area (% coronary artery stenosis) was calculated, and the mean of the three sections was considered to be the degree of coronary atherosclerosis of each animal.

Measurement of serum NOx levels

Serum NOx levels were determined using an automatic analyzer that employs automated flow injection analysis (TCI-NOX5000S, Tokyo Kasei Kogyo, Tokyo) as described previously (19, 20). Briefly, serum samples were centrifuged at 3,000 g for 10 min. The supernatant (0.1 ml) was diluted with 0.4 ml of distilled water and 0.3 ml of 0.3 N NaOH was added. After incubation for 5 min at room temperature, 0.3 ml of 5% (w/v) ZnSO₄ was added and incubated for a further 5 min. The mixture was centrifuged at 2,800 g for 10 min and the supernatant was applied to the analyzer. Nitrite reacts with a Griess reagent and forms a purple azo compound. The absorbance at 540 nm is measured. Nitrate is determined by reducing it to nitrite through a copperized cadmium reduction column.

We tested the reproducibility and the recovery using the normolipemic serum (from a rabbit of ND group, total cholesterol 79 mg/dl) and the hyperlipemic serum (from a rabbit of CD group, total cholesterol 1,593 mg/dl). The coefficient of variation was 8.1% in the normolipemic serum (n = 7) and 3.1% in the hyperlipemic serum (n = 7). The recovery rates of nitrate from the normolipemic (n = 6) and the hyperlipemic serum (n = 6), both added 40 μM nitrate, were 98.7 ± 10.1% and 108.5 ± 4.9%, respectively.

Statistics

The data were analyzed using one-factor analysis of variance. If a statistically significant effect was found, Newman-Keuls' test was performed to isolate the differ-
ence between the groups. A $p$ value of less than 0.05 was considered significant. The values in the text, tables and figures are expressed as means ± SEM.

**Results**

**Body weight, blood pressure and blood chemistry**

Body weight and systolic blood pressure during the experiments are shown in Table 1. Body weight increased similarly in the three groups throughout the experimental period. There was no significant difference in systolic blood pressure among the groups, except that at 3 months, systolic blood pressure was significantly higher in CD than in CD+E.

Estrogen replacement significantly increased serum estradiol concentration compared to the other two groups (ND, 4.1 ± 0.7; CD, 1.9 ± 0.6; CD+E, 12.0 ± 2.7 pg/ml, $p<0.05$).

As shown in Table 2, 1% cholesterol diet significantly increased serum total cholesterol and TBARS levels, and decreased HDL cholesterol levels. Estrogen replacement, however, did not affect serum total cholesterol, HDL cholesterol and TBARS levels. There was no significant difference in serum triglyceride levels among the three groups.

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Table 1. Body Weight (BW) and Systolic Blood Pressure (SBP) during the Study.

<table>
<thead>
<tr>
<th>Month</th>
<th>ND</th>
<th>CD</th>
<th>CD+E</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (kg) 0</td>
<td>3.0±0.1</td>
<td>3.1±0.1</td>
<td>3.1±0.1</td>
</tr>
<tr>
<td>1</td>
<td>3.3±0.1</td>
<td>3.5±0.1</td>
<td>3.4±0.1</td>
</tr>
<tr>
<td>3</td>
<td>3.9±0.1</td>
<td>3.8±0.1</td>
<td>4.0±0.1</td>
</tr>
<tr>
<td>SBP (mmHg) 0</td>
<td>83.0±1.2</td>
<td>83.5±1.7</td>
<td>82.0±0.8</td>
</tr>
<tr>
<td>1</td>
<td>84.0±2.3</td>
<td>86.7±1.1</td>
<td>83.1±1.0</td>
</tr>
<tr>
<td>3</td>
<td>84.8±1.4</td>
<td>88.0±1.5*</td>
<td>83.1±1.0</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM.
ND: normal diet group, CD: cholesterol diet group, CD+E: cholesterol diet and estrogen replacement group.
* $p<0.05$ vs. CD+E

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Table 2. Serum Lipid Concentration.

<table>
<thead>
<tr>
<th>ND</th>
<th>CD</th>
<th>CD+E</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-Chol (mg/dl) 86±17</td>
<td>1611±123**</td>
<td>1554±111***</td>
</tr>
<tr>
<td>HDL-Chol (mg/dl) 31.0±2.9</td>
<td>18.0±3.1**</td>
<td>1.6±2.3***</td>
</tr>
<tr>
<td>Triglyceride (mg/dl) 64.8±8.5</td>
<td>62.2±16.1</td>
<td>39±6.1</td>
</tr>
<tr>
<td>TBARS (nmol/ml) 0.98±0.04</td>
<td>3.38±0.18**</td>
<td>3.44±0.12**</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM.
* $p<0.01$ vs. ND

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Atherosclerosis in the aorta and coronary artery

Oil red O-positive area in the aortas was not observed in ND, while it was distinct in CD and in CD+E (Fig. 1). The Oil red O-positive area was, however, significantly greater in CD than in CD+E as shown in Fig. 2A (CD, 17.3±2.2; CD+E, 9.3±0.8%, $p<0.05$).

Coronary atherosclerosis was not observed in ND. As shown in Fig. 2B, stenosis of the left main coronary artery was significantly greater in CD than in CD+E (CD, 30.6±
We also examined the epicardial coronary segments, e.g., right coronary, midportion of left anterior descending and circumflex. However, we could not evaluate the degree of the stenosis because atherosclerosis was scarce in these segments.

**Serum NOx level**

It is worth noting that, serum NOx levels were significantly lower in CD than in ND as shown in Fig. 3 (ND, 37.6±3.6 μM; CD, 25.3±3.1 μM, p < 0.05). Serum NOx levels in CD+E were higher than in CD although the difference was not significant (CD+E, 34.4±3.8 μM, p = 0.08).

**Discussion**

We showed that estrogen replacement to ovariectomized rabbits inhibited the cholesterol diet-induced atherosclerosis in both the aorta and the coronary artery without affecting serum lipid levels. The serum NOx levels were decreased by cholesterol feeding and there was a non-significant trend towards higher NOx levels after estrogen replacement.

We found a number of animal experiments in which estrogen inhibited atherosclerosis induced by a high cholesterol diet. Some experiments focused on the beneficial effects of estrogen on the lipid metabolism (21-23), and others pointed out additional mechanisms such as direct actions of estrogen on the vascular wall (24-26). Since estrogen replacement had no effect on the serum lipid levels, estrogen effects on atherosclerosis were independent of lipid metabolism in this study. Although it is unknown why estrogen administration did not affect the serum lipid levels in this study, several conditions related to the experiments may be the cause of the difference compared to the previous studies (21-23): Kushwaha et al. (22) and Adams et al. (23) examined the effects of estrogen using monkeys, while we used rabbits. Kushwaha et al. (21) also reported that exogenous estrogen attenuated hypercholesterolemia in rabbits. However, in their study the rabbits were fed with a lower cholesterol diet (0.2%) and were injected with a higher dose of estrogen (500 mg/kg/wk) than in our study. Thus the lower cholesterol diet or the higher estrogen administration may affect serum lipid levels. It has been reported that estrogen inhibits LDL oxidation in vitro (27) and in vivo (28). These studies indicate that estrogen has an anti-oxidant action and may inhibit atherosclerosis. However, it is unlikely that estrogen inhibited the lipid peroxidation in this study because serum levels of TBARS, an index of lipoprotein oxidation, were not changed by estrogen replacement.

Although hypertension is reported to increase after menopause (29), the influence of estrogen administration on blood pressure is controversial (30). In the present study, systolic blood pressure in the cholesterol diet group was significantly higher by ≈5 mmHg than in the cholesterol diet plus estrogen replacement group at 3 months into the experiment. Although the difference in blood pressure between the two groups was small, estrogen might have decreased blood pressure and influenced atherosclerosis.

In the present study, serum NOx levels were significantly decreased by cholesterol feeding and partially restored by estrogen replacement. These results are consistent with previous studies (13, 31). Tanaka et al. (31) reported that plasma NOx levels were lower in hypercholesterolemic patients, and Rosselli et al. (13) reported that plasma NOx levels were increased by estrogen replacement therapy in postmenopausal women. Since NO is a labile substance, it is convenient to measure NOx as a marker of NO production (19, 20). We collected blood after 16-hour fasting to exclude the influence of food intake on serum NOx levels (32, 33). Furthermore, as is shown in the methods, the accuracy of the assay was not influenced by high cholesterol concentration of the serum. This indicates that serum NOx levels reflected the endogenous production of NO. However, the origin of circulating NO is obscure because NO is synthesized not only in endothelial cells but also in other cells and tissues (34). It is known that NO inhibits atherosclerosis by regulating several factors, e.g., cell adhesion (35) and smooth muscle cell proliferation (36). Thus, augmented production of NO by estrogen may affect atherosclerosis via these factors.

Other vascular effects unrelated to NO might be involved in the mechanism of atherogenesis. Fischer-Dzoga et al. (37) reported that estrogen inhibited the growth of cultured smooth muscle cells from rabbit aorta induced by hyperlipemic serum. Fischer et al. (38) reported that estrogen decreased collagen synthesis in atherosclerotic rabbit aortas. Consequently, it is possible that estrogen directly inhibited smooth muscle cell prolifera-

![Fig. 3. Bar graph showing the serum nitrite/nitrate (NOx) levels in the normal diet group (ND), cholesterol diet group (CD), and cholesterol diet and estrogen replacement group (CD+E).](image-url)
tion and/or collagen synthesis although no direct evidence was shown in this study.

In conclusion, estrogen replacement inhibited atherosclerosis formation in cholesterol-fed ovariectomized rabbits. Direct actions on the vascular wall including NO production could contribute to the effects of estrogen.

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